

H₂ fueled ATP synthesis on an electrode: mimicking cellular respiration

Óscar Gutiérrez-Sanz, Paolo Natale, Ileana Márquez, Marta C. Marques, Sonia Zacarías, Marcos Pita, Inês A. C. Pereira, Iván López-Montero*, Antonio L. De Lacey*, Marisela Vélez*,

Dr.O. Gutiérrez-Sanz, Dr. I. Márquez, Dr. M. Pita, Dr. A. L. De Lacey and Dr. M. Vélez,

Instituto de Catálisis y Petroleoquímica, CSIC

c/ Marie Curie 2, 28049 Madrid (Spain)

E-mail: marisela.velez@icp.csic.es

Dr. M. C. Marques, S. Zacarias, Dr. I. A. C. Pereira

Instituto de Tecnologia Quimica e Biologica

Universidade Nova de Lisboa

Apartado 127, 2781-901 Oeiras (Portugal)

Dr. Paolo Natale and Dr. Iván López Montero

Universidad Complutense de Madrid

Avda. Complutense s/n 28040 Madrid

Instituto de Investigación i+12 hospital 12 de Octubre

Avda. Córdoba s/n 28041 Madrid

Abstract

ATP, the molecule used by living organisms to supply energy to many different metabolic processes, is synthesized mostly by the ATPase synthase using a proton or sodium gradient generated across a lipid membrane. We present evidence that a modified electrode surface integrating a NiFeSe hydrogenase and a F₁F_o-ATPase in a lipid membrane can couple the electrochemical oxidation of H₂ to the synthesis of ATP. This electrode-assisted conversion of H₂ gas into ATP could serve to generate this biochemical fuel locally when required in biomedical devices or enzymatic synthesis of valuable products.

Introduction

Nature uses wise strategies to manage energy conversion between different sources that would be interesting to mimic artificially for their use in the development of new man-made devices. At cellular and molecular level the transduction of energy is linked to the vectorial transfer of ions across a membrane ^[1]. The most typical gradient present in biological energy transfer is the proton gradient. During respiration and photosynthesis, using energy from either chemical oxidation or light, respectively, a proton gradient is created between two compartments separated by a lipid bilayer. The energy stored as an electrochemical gradient is invested into several key biochemical processes: the transfer of certain biomolecules against a concentration gradient^[2], the chemical synthesis of ATP^[3], or the rotation of bacterial flagella ^[4]. Particularly interesting is the case where the proton gradient is used to drive the enzyme F1Fo-ATPase to produce ATP, the molecule that will convey this “captured energy” to many metabolic processes required by the cell.

In the quest to use nature’s wisdom to develop new devices, the ability to harness and transduce energy at the molecular level is a big challenge. Great progress in immobilizing enzymes by different strategies has allowed the exploitation of their substrate specificity to catalyse chemical reactions for industrial applications^[5] and to develop biosensors with great selectivity and capacity to detect analytes ^[6]. However, to mimic and utilize the vectorial ion movement required for the generation of electrochemical gradients in living cells demands two issues: spatial control over the assembly of the participating proteins^[7], and the presence and maintenance of two independent ion-impermeable compartments^[8]. Liposomes ^[9], where a lipid bilayer separates the inner content from the outer solution, have been used as model systems to couple light energy to different biochemical reactions. The proton gradient developed by a light sensitive component, either bacteriorhodopsin or artificial photosynthetic reaction centres, has been coupled to Ca^{2+} active transport ^[10], to ATP synthesis ^[9b] or to activate bacterial pumps^[11]. But liposomes are fragile, and increasing robustness is desired for *in vitro* applications that require coupling artificially generated electrochemical gradients to solid transducers, such as lab-scale biosensors and biomimetic fuel cells. A strategy to improve the robustness of

liposomes that maintain proton gradients generated by light, cytochrome oxidase or by F1Fo-ATPase has been to deposit the lipid membranes on different solid surfaces: silica particles^[12], polymersomes^[13], electrode surfaces^[14] and nanowires^[15]. In addition to increased stability, a challenging improvement is coupling different energy sources, such as electricity and molecular hydrogen (H₂), to ATP synthesis in artificial systems.

In this work we present a new set-up where H₂ is used to fuel ATP synthesis on an electrode surface modified to include two oriented proteins and a lipid membrane. Previously, we demonstrated that a proton gradient can be produced by electroenzymatic H₂ oxidation on an electrode surface covered by a phospholipid bilayer (PhBL).^[16] We now show that this proton gradient can be harnessed to synthesise ATP when a membrane bound NiFeSe hydrogenase and an ATPase are co-immobilized on a flat gold electrode surface in the presence of a lipid membrane (Figure 1).

The assembly process was followed using Atomic Force Microscopy (AFM) and Quartz Crystal Microbalance (QCM), for characterizing the biomimetic construction, and electrochemistry combined with UV- spectroscopy, for evaluating its functional properties. The assembly was done sequentially, following the steps previously described to covalently bind the hydrogenase on the electrode surface with the appropriate orientation for direct electron transfer^[16a, 17]. Once this first protein layer was characterized, the proteoliposomes containing the *E.coli* F1Fo ATPase were incubated overnight in the presence of polystyrene beads to remove excess detergent and allow their fusion to the surface.

The AFM images (Figure 2 A,B,C), taken on flat annealed gold substrates prepared in parallel to the gold wires used for the electrochemical measurements, confirm that each modification step changed the surface as expected. Figure 2A reflects the typical terraces found on flame annealed gold. Figure 2B illustrates the appearance of a hydrogenase monolayer with a surface roughness of 5 nm after its covalent binding to the gold surface modified with a self-assembled monolayer (SAM) of 4-aminothiophenol. This roughness corresponds to that expected for a monolayer of hydrogenase molecules considering the enzyme dimensions^[18]. Fig 2C illustrates that fusion of the proteoliposomes containing ATPase smooths the underlying

surface roughness but shows protrusions corresponding to the membrane embedded ATPase. The fact that the protrusions are only seen on top of the bilayer indicates that the proteins are inserted with only one orientation, with the large F1 soluble domain sitting away from the substrate. This oriented protein insertion on lipid surfaces when using detergent destabilized bilayers has been previously reported^[19]. Fig S1 in supplementary material shows the height profile of the different surfaces shown in Fig 2 A-C.

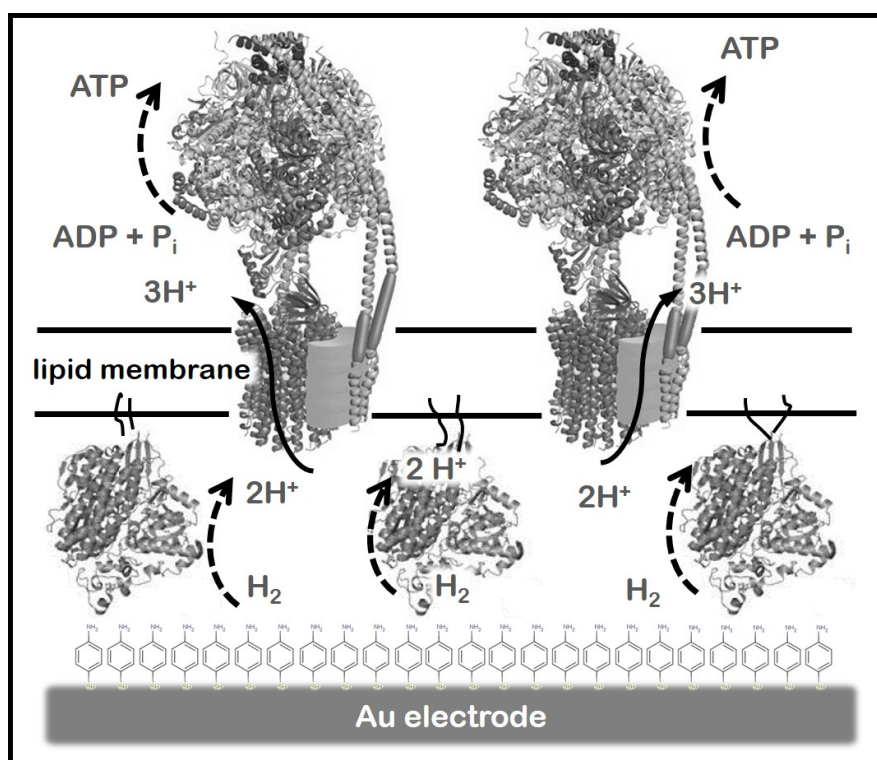


Figure 1. A) Schematic representation of the supramolecular construction to synthesize ATP from enzymatic H_2 electrooxidation. NiFeSe Hydrogenase (Hase) immobilized covalently on an Au electrode modified with a SAM of 4-aminothiophenol and anchored to a PhBL through its lipid tail^[16a]. The PhBL embeds F1Fo ATPases. Protons (H^+) produced from H_2 electrooxidation are used to synthesize ATP from ADP and inorganic phosphate (Pi).

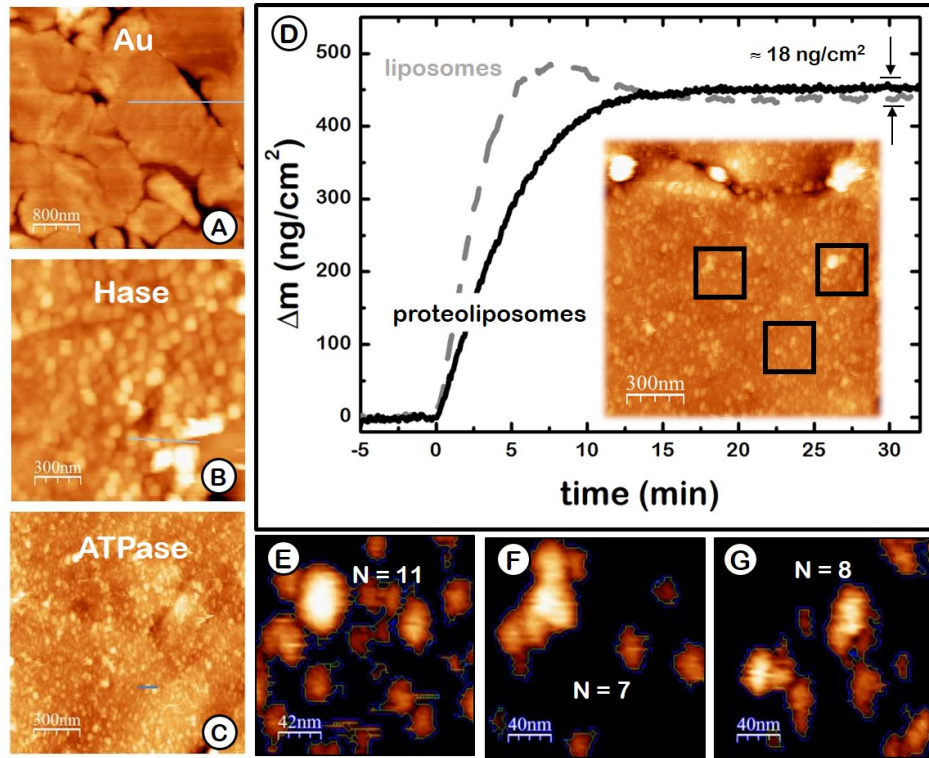


Figure 2. A-C) AFM topography images of A) the bare annealed gold surface, B) the Hase monolayer and C) the F1Fo ATPase reconstituted into the PhBL spread on the Hase monolayer. **D)** QCM monitoring of liposomes (dashed line) and F1Fo proteoliposomes (solid line) adsorption to a SiO_2 surface. **Inset:** AFM image of an F1Fo ATPase containing PhBL. **E-G)** Three regions from AFM image in D) used to estimate the average number of proteins protruding more than 5 nm ($N = 9$ per $200 \times 200 \text{ nm}^2$).

Panel D in Figure 2 presents the surface density evolution obtained with the QCM when F1Fo ATPase proteoliposomes, of the same composition used for covering the gold electrodes, were fused over a SiO_2 surface. The difference in mass detected after fusing liposomes, with and without proteins, and the number of protrusions detected per unit area on the AFM images were used to estimate the amount of protein present on the modified electrodes. SiO_2 surfaces modified with proteoliposomes show an increase in mass of $18 \text{ ng}/\text{cm}^2$. The average number of protrusions per cm^2 estimated from the AFM images is 2×10^{10} . However, their size is not homogeneous and is larger than expected for individual molecules, indicating that each protrusion is likely to include more than one protein^[20]. The MW of the F1Fo ATPase is $\approx 500 \text{ kD}$, so the expected mass increase for the average number of protrusions observed, is indeed $\approx 18 \text{ ng}/\text{cm}^2$. As the protrusions are not single proteins, this surface concentration would represent a lower limit of proteins present on the surface. The QCM registered the fusion of

proteoliposomes directly onto a SiO₂ surface. However, the amount of protein on the gold surface observed with the AFM is clearly larger. A more convenient way to estimate the mass content is to evaluate the percentage of the area covered by the protein from the AFM images. This estimate indicates that 30-40% of the surface is covered by protein. Considering that a compact layer of F1Fo ATPase would give approximately 1000 ng/cm² (the weight of the proteins that fit in one cm² if densely packed) the amount of protein on the gold surface would be around 350 ng/cm². This information will be used below to estimate the turnover rate of the F1Fo ATPase on the electrode.

In order to study the functionality of the fully modified electrode including both enzymes, we activated the Hase in an anaerobic chamber under 1 atm of H₂ and subsequently performed a cyclic voltammogram sweeping from -0.6 to +0.2 V to check the bioelectrocatalytic response in phosphate buffer. The increase of the anodic current that reaches a plateau at -0.2 V is attributed to the oxidation of H₂ to protons (Figure 3A)^{13, 14}. In the following experiment (Figure 3B) protons were generated at the PhBL/electrode interface of the biomimetic construction by applying a continuous potential of +150 mV vs. SCE during 130 min in the presence of 500 μM ADP, under 1 atm of H₂ and stirring conditions. Aliquots were taken periodically from the bulk solution to measure the ATP concentration by a spectrophotometric method^[21]. Control measurements were recorded with PhBL-modified electrodes in the absence of either Hase or F1Fo ATPase (Figure 3B). The experiments showed that ATP production is detected in the bulk solution during hydrogen oxidation catalyzed by the immobilized hydrogenase only when F1Fo ATPase, ADP and phosphate are present. ATP detection was negligible in the control experiments performed with electrodes incorporating only one enzyme, either ATPase from the proteoliposomes deposited directly on the gold electrode surface, or the hydrogenase covalently bound to the electrode and covered by the PhBL as reported previously^[16a]. We also observe that at longer times, the rate of ATP production decreases. It is likely that the long term exposure to H₂ at 37°C, conditions at which the experiment is performed, favour the desorption of the monolayer, affecting therefore the orientation and availability of the hydrogenase. Furthermore, the positive potential at which the

experiments are run could also cause certain extent of hydrogenase inactivation. Both effects could be contributing to the decrease in ATP production observed at long times.

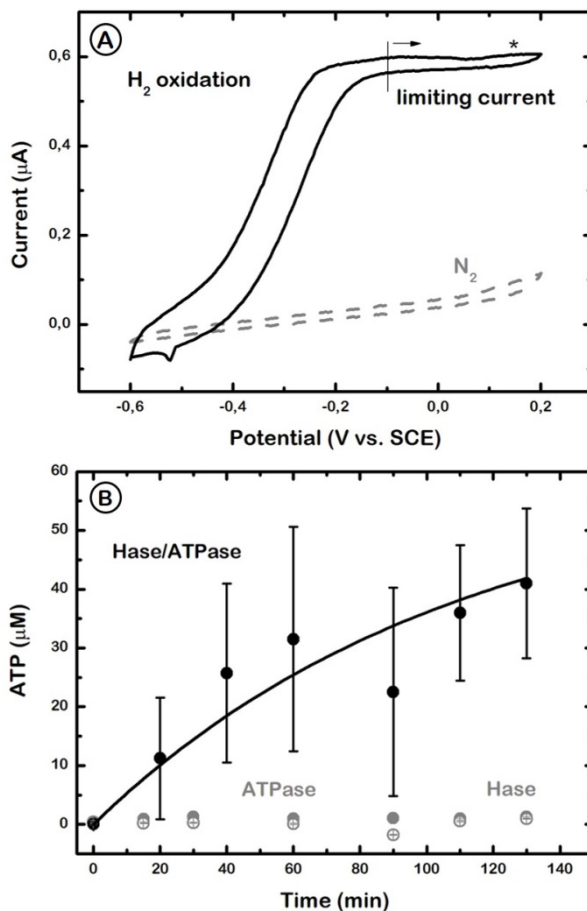


Figure 3. **A)** Cyclic voltammograms of the Hase/PhBL/ATPase modified electrode in 0.1 M phosphate buffer (pH 8.0) after Hase activation through H_2 incubation (solid line) or under N_2 before activation (dashed line). The star indicates the redox potential (150 mV vs. SCE) applied to drive ATP production. Scan rate = 0.01 Vs^{-1} . Temperature = 30°C . **B)** ATP synthesis from ADP and phosphate in 0.1 M phosphate buffer (pH 8.0) at 150 mV and under 1 atm H_2 . ATP concentration in the bulk solution is shown as a function of time for Hase/PhBL/ATPase (black solid circles), PhBL/ATPase (gray solid circles) and Hase/PhBL (gray circles) electrodes. Error bar represents the standard deviation of three measurements made from different electrode preparations.

The value for the turnover rate of ATP synthesis measured is strongly dependent on the exact experimental setup and conditions. Reported values range from $40\text{--}210 \text{ ATP s}^{-1}$ observed on liposomes energized by an acid-base transition^[22] to 7 ATP s^{-1} measured for the light-driven protein inserted in liposomes^[9b], or the much smaller $1.5 \times 10^{-4} \text{ ATP s}^{-1}$ turnover rate detected when the proton gradient is produced by bacteriorhodopsin in polymerosomes^[13]. In our experiments, considering that the amount of protein on the flat gold surface is 350 ng/cm^2 , the

average ATP synthase turnover rate is around 1800 s^{-1} , much higher than values previously reported. One possible explanation is that the hydrogenase high turnover rate (up to 900 s^{-1} for H_2 oxidation)^[23] and the rapid diffusion of H_2 across lipid membranes allows a quick and constant establishment of a proton gradient at the electrode interface,^[16a] even if the supported PhBL may leak protons due the presence of defects in its configuration ^[16b] This net proton gradient across the PhBL of over one pH unit^[16a] in the adequate direction is sufficient to drive the reconstituted F1Fo ATPase, in the configuration of the scheme of Figure 1, to produce ATP from ADP and phosphate. An additional consideration is that, as the activity measurements were performed on gold wires and not flat surfaces, it could be that the F1Fo ATPase density on the wire is larger, which would mean that each individual protein has a smaller turnover than this estimate.

This estimated turnover rate can only be considered as an indication that the enzyme has a higher turnover when fuelled with H_2 than the previously reported ones when energized using light or acid-base transitions. The exact amount of protein and confirmation about its orientation would be needed in order to obtain a reliable turnover rate to confirm this observation.

We also tested the capacity of the F1Fo ATPase incorporated into the lipid bilayer fused on an electrode to hydrolyse ATP and to generate a proton gradient in the absence of the hydrogenase (Figure 4A). Pyrophosphate detection in the bulk solution by a spectrophotometric method was used to measure ATP hydrolysis (Figure 4B) and the proton gradient was detected by Differential Pulse Voltametry (DPV) following the shift in the oxidation peak of the SAM over the Au surface upon changes of the local pH at the electrode/PhBL interface (Figure 4C).

[16a]

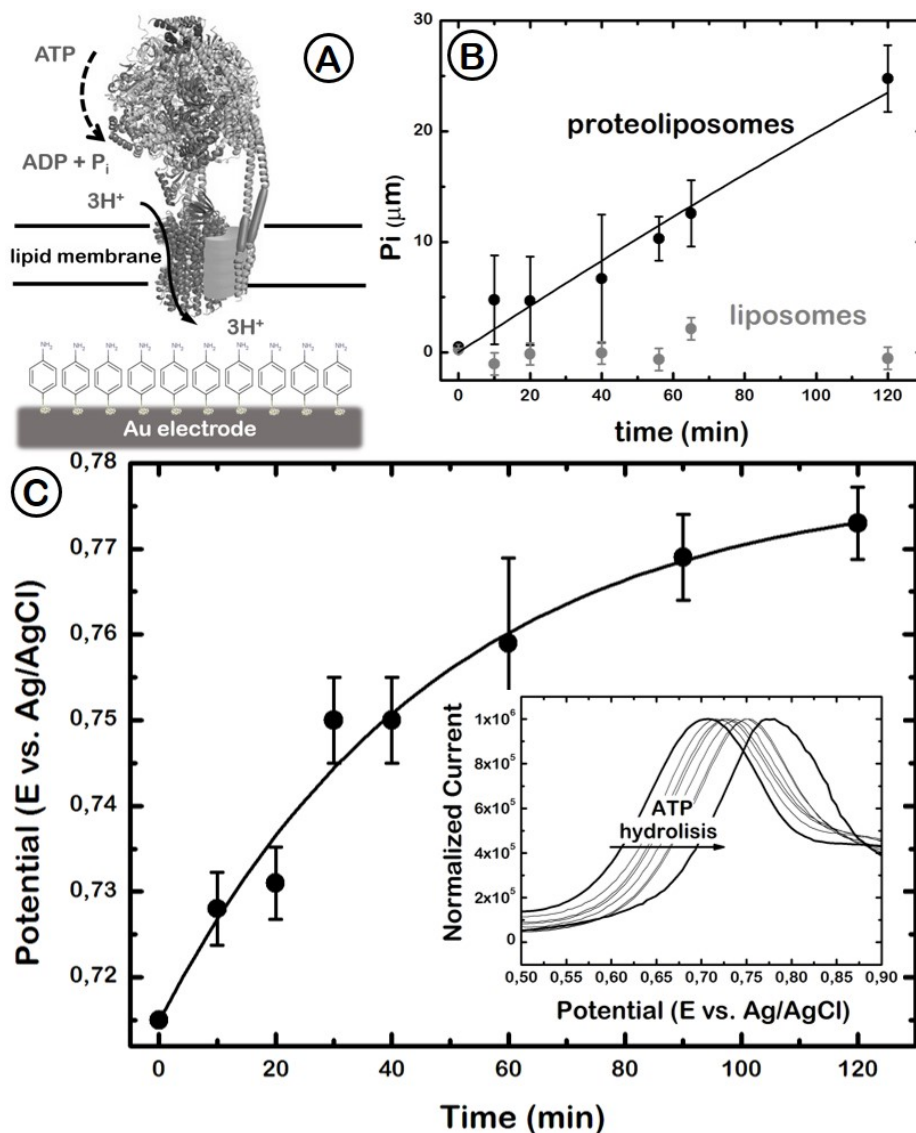


Figure 4. **A)** Schematic representation of the PhBL reconstituted with F1Fo-ATP synthase over an Au electrode. The ATP hydrolysis by F1Fo leads to proton pumping across the membrane, which results in a decrease of the pH of the aqueous compartment between the lipid membrane and the Au electrode surface. **B)** ATP hydrolysis monitored by phosphate production as a function of time for a F1Fo-proteoliposomes modified electrode (black solid circles) and for a bare liposomes modified electrode (gray solid circles). Initial ATP concentration was 150 μM and temperature was 37°C. **C)** The simultaneous proton pumping activity of F1Fo-ATP synthase is monitored over time by Differential Pulse Voltammetry (inset). The oxidation peak potential of the SAM on Au is shown as a function of time after ATP hydrolysis started. Error bar represents the standard deviation of two independent measurements.

The electrode modified with F1Fo ATPase proteoliposomes was incubated in Tris HCl Buffer at 37°C, pH 8. ATP was added to the solution and the Pi concentration was monitored during 120 minutes, observing a nearly linear increase (Fig 4B). The same experiment carried out with an electrode modified with just the liposomes, where ATPase is absent, did not yield any significant amount of Pi (Fig. 4B).

In parallel, the pH at the electrode/PhBL interface was measured using a procedure previously described^[16a]. The SAM present on the gold surface can act as pH sensitive probe because its redox potential is pH-dependent. The proteoliposomes-modified electrode was submitted to 15 cyclic voltammetry scans between 0 and 1 V vs. Ag/AgCl, causing a partial dimerization of the aminophenol derivatives. After incubating the electrode in the ATP solution, the shift in the oxidation peak of the dimerized 4- aminothiophenol measured by DPV is attributed to the pH change at the electrode/PhBL interface associated with the ATP hydrolysis activity of the reconstituted F1Fo ATPase. The result obtained from two independent experiments shown in Figure 4C indicates a peak potential shift from 715 to 775 mV. This +60 mV shift corresponds to one pH unit^{13a} and stabilizes after 60 minutes of incubation, whereas the Pi detected shows an apparent linear production up to 120 minutes (Fig. 4). The hydrolysis rate measured is $0.28 \mu\text{M min}^{-1}$, which gives a turnover rate of 788 ATP s^{-1} . The reason for the stabilization of the pH gradient at longer times, in spite of the linear increase in Pi production is not clear. It could be that a membrane potential builds up across the lipid bilayer, opposing further proton translocation.

It is not the first time that a membrane bound F1Fo ATPase is reconstituted onto an electrode surface^[14] and that the proton gradient generated during ATP hydrolysis is monitored electrochemically. In^[14b, 14c], the translocation of protons across the lipid film was investigated using square wave voltammetry (SWV), double potential-pulse chronoamperometry (CA) or impedance spectroscopy with the aim of correlating the activity of the enzyme with electrochemical processes occurring at the gold electrode. The authors were able to demonstrate that it was possible to measure ion transport through a protein as a function of the membrane potential, which is difficult to measure on other reconstituted systems. However, in our setup we were able to go beyond the

state of the art in two ways: we have managed to measure directly the pH shift generated on the proximity of the electrode surface due to the ATPase activity, and we have managed to synthesize ATP by coupling the F₁F₀ ATPase activity with the proton gradient generated by the bioelectrooxidation of H₂ at the PhBL/interface. We show that the reconstituted F₀F₁ ATPase is reversible, capable of synthesizing ATP in the presence of a proton gradient, or generating a proton gradient upon ATP consumption.

In summary, we present evidence that a modified electrode integrating a NiFeSe hydrogenase, a F₀F₁-ATPase and a lipid membrane can serve to couple the electrochemical H₂ oxidation to ATP synthesis. Electrode assisted interconversion between these two valuable and versatile fuels could open new possibilities for using H₂ gas to locally generate ATP needed for reactions in biomedical devices or enzymatic organic synthesis of valuable products. Although oxygen sensitivity of hydrogenases imposes important restrictions for the development of practical devices in a laboratory environments, the high turnover of electroenzymatic H₂ oxidation allows a much faster proton gradient formation across the membrane as compared to those generated by light^[9b, 12a]. Therefore, this bi-enzymatic system will still be advantageous to artificially fuel ATP synthesis.

Experimental Section

The NiFeSe Hase from *Desulfovibrio vulgaris* was isolated and purified as reported previously^[23-24]. F₁F₀ATPase from *E. coli* was purified as described in the *Supplementary Material*. Au electrodes of 0.16 cm² surface area were prepared and modified with 4-aminothiophenol SAM as described previously^[25]. Au/4-

aminothiophenol SAM /Dv-SeHase/PhBL/ATPase electrodes were constructed by incubating, sequentially, the gold electrode on a 4-aminothiophenol solution, 12 mM solution of Dv-SeHase, in 0.1M phosphate buffer (pH 5.5) with 0.1% n-dodecyl- β -D-maltoside, (DDM) at 48° C. The electrodes were then incubated for 30 min in a 21mM solution of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride in 50 mM Tris-HCl buffer (pH 6.0) with 0.1% DDM. Finally, the electrodes were incubated overnight in a 0.2 mgml⁻¹ proteoliposome suspension in the presence of 240 mgml⁻¹ Calbiosorb adsorbent biobeads (Calbiochem). Electrochemical, AFM and QCM measurements were carried out as reported previously^[16a, 17, 26]. Inorganic phosphate (Pi) determination was performed by the Green Malachite assay^[27]. ATP concentration was measured using the Luciferase assay kit from Molecular Probes. For more details see the supplementary material.

Acknowledgements: This research was funded by the Spanish MINECO (project CTQ2012-32448) and by the Fundação para a Ciência e a Tecnologia (project PTDC/BBB-EP/0934/2012 and UID/Multi/04551/2013). O.G.-S. thanks MINECO for an FPI grant.

ILM acknowledges the European Research Council (ERC), grant no. ERC-StG-2013-338133 titled “mitochon” and the “Ramón y Cajal” program (RyC-2013-12609) from the Spanish Ministry of Economy. PN acknowledges Dr. rer. nat. Gabriele Deckers-Hebestreit (Universität Osnabrück) for the generous gift of F1 β -subunit and F0-b subunit specific antibodies.

- [1] S. F. David G. Nicholls, *Bioenergetics 4*, Academic Press, **2013**.
- [2] Y. Shi, *Annual Review of Biophysics* **2013**, 42, 51-72.
- [3] W. Junge, N. Nelson, *Annual Review of Biochemistry* **2015**, 84, 631-657.
- [4] aT. Minamino, K. Imada, *Trends in Microbiology* **2015**, 23, 267-274; bS. Kojima, *Current Opinion in Microbiology* **2015**, 28, 66-71.
- [5] C. Garcia-Galan, Á. Berenguer-Murcia, R. Fernandez-Lafuente, R. C. Rodrigues, *Advanced Synthesis & Catalysis* **2011**, 353, 2885-2904.
- [6] A. P. F. Turner, *Chemical Society Reviews* **2013**, 42, 3184-3196.

- [7] D. G. G. McMillan, S. J. Marritt, M. A. Firer-Sherwood, L. Shi, D. J. Richardson, S. D. Evans, S. J. Elliott, J. N. Butt, L. J. C. Jeuken, *Journal of the American Chemical Society* **2013**, *135*, 10550-10556.
- [8] S. Bhattacharya, M. Schiavone, A. Nayak, S. K. Bhattacharya, *Biotechnology and Applied Biochemistry* **2004**, *39*, 293-301.
- [9] aG. Steinberg-Yfrach, P. A. Liddell, S.-C. Hung, A. L. Moore, D. Gust, T. A. Moore, *Nature* **1997**, *385*, 239-241; bG. Steinberg-Yfrach, J.-L. Rigaud, E. N. Durantini, A. L. Moore, D. Gust, T. A. Moore, *Nature* **1998**, *392*, 479-482.
- [10] I. M. Bennett, H. M. V. Farfano, F. Bogani, A. Primak, P. A. Liddell, L. Otero, L. Sereno, J. J. Silber, A. L. Moore, T. A. Moore, D. Gust, *Nature* **2002**, *420*, 398-401.
- [11] aA. Verchère, I. Broutin, M. Picard, *Scientific Reports* **2012**, *2*, 306; bD. Wendell, J. Todd, C. Montemagno, *Nano Letters* **2010**, *10*, 3231-3236.
- [12] aT.-J. M. Luo, R. Soong, E. Lan, B. Dunn, C. Montemagno, *Nat Mater* **2005**, *4*, 220-224; bG. Nordlund, J. B. Sing Ng, L. Bergström, P. Brzezinski, *ACS Nano* **2009**, *3*, 2639-2646.
- [13] H.-J. Choi, C. D. Montemagno, *Nano Letters* **2005**, *5*, 2538-2542.
- [14] aR. Naumann, A. Jonczyk, R. Kopp, J. van Esch, H. Ringsdorf, W. Knoll, P. Gräber, *Angewandte Chemie International Edition in English* **1995**, *34*, 2056-2058; bR. Naumann, A. Jonczyk, C. Hampel, H. Ringsdorf, W. Knoll, N. Bunjes, P. Gräber, *Bioelectrochemistry and Bioenergetics* **1997**, *42*, 241-247; cR. Naumann, T. Baumgart, P. Gräber, A. Jonczyk, A. Offenhäusser, W. Knoll, *Biosensors and Bioelectronics* **2002**, *17*, 25-34.
- [15] aC. Ge, K. S. Orosz, N. R. Armstrong, S. S. Saavedra, *ACS Applied Materials & Interfaces* **2011**, *3*, 2677-2685; bT. W. McBee, L. Wang, C. Ge, B. M. Beam, A. L. Moore, D. Gust, T. A. Moore, N. R. Armstrong, S. S. Saavedra, *Journal of the American Chemical Society* **2006**, *128*, 2184-2185.
- [16] aÓ. Gutiérrez-Sanz, C. Tapia, M. C. Marques, S. Zacarias, M. Vélez, I. A. C. Pereira, A. L. De Lacey, *Angewandte Chemie International Edition* **2015**, *54*, 2684-2687; bO. Gutiérrez-Sanz, M. Marques, I. A. C. Pereira, A. L. De Lacey, W. Lubitz, O. Rüdiger, *The Journal of Physical Chemistry Letters* **2013**, *4*, 2794-2798.
- [17] C. Gutiérrez-Sánchez, D. Olea, M. Marques, V. M. Fernández, I. A. C. Pereira, M. Vélez, A. L. De Lacey, *Langmuir* **2011**, *27*, 6449-6457.
- [18] M. C. Marques, R. Coelho, A. L. De Lacey, I. A. C. Pereira, P. M. Matias, *Journal of Molecular Biology* **2010**, *396*, 893-907.
- [19] P.-E. Milhiet, F. Gubellini, A. Berquand, P. Dosset, J.-L. Rigaud, C. Le Grimmellec, D. Lévy, *Biophysical Journal* **2006**, *91*, 3268-3275.
- [20] F. Minauro-Sanmiguel, S. Wilkens, J. Garcia, J. P. D. Boyer, *Proceedings of the National Academy of Sciences of the United States of America* **2005**, *102*, 12356-12358.
- [21] S. Karamohamed, G. Guidotti, *Biotechniques* **2001**, *31*, 420-425.
- [22] S. Fischer, P. Gräber, P. Turina, *Journal of Biological Chemistry* **2000**, *275*, 30157-30162.
- [23] F. A. Valente, A. S. Oliveira, N. Gnadt, I. Pacheco, A. Coelho, A. Xavier, M. Teixeira, C. Soares, I. C. Pereira, *J Biol Inorg Chem* **2005**, *10*, 667-682.
- [24] aO. Gutiérrez-Sanz, M. Marques, C. A. Baltazar, V. Fernández, C. Soares, I. C. Pereira, A. De Lacey, *J Biol Inorg Chem* **2013**, *18*, 419-427; bC. d. K. van der Does, J.; van der Laan, M.; Driessen, A.J., *Methods in Enzymology* **2003**, *372*, 86-98.
- [25] O. Gutiérrez-Sanz, D. Olea, M. Pita, A. P. Batista, A. Alonso, M. M. Pereira, M. Vélez, A. L. De Lacey, *Langmuir* **2014**, *30*, 9007-9015.
- [26] P. Mateos-Gil, I. Márquez, P. López-Navajas, M. Jiménez, M. Vicente, J. Mingorance, G. Rivas, M. Vélez, *Biochimica et Biophysica Acta (BBA) - Biomembranes* **2012**, *1818*, 806-813.
- [27] P. A. Lanzetta, L. J. Alvarez, P. S. Reinach, O. A. Candia, *Analytical Biochemistry* **1979**, *100*, 95-97.

